

NOVEL INTERLEUKIN-1 RECEPTOR
INTRACELLULAR LIGAND PROTEINS
AND INHIBITORS OF LIGAND BINDING

BACKGROUND OF THE INVENTION

10 The present invention relates to the field of anti-inflammatory substances and other substances which act by inhibiting binding to the intracellular domain of an interleukin-1 receptor (hereinafter "IL-1-R"), such as, for example, the p80, type I IL-1 receptor. More particularly, the present invention is directed to novel ligands which bind to the IL-1-R intracellular domain and to inhibition or modulation of
15 signal transduction by this receptor.

Interleukin-1- α and interleukin-1- β (herein collectively "IL-1") are cytokines which produce a wide range of cellular activities. IL-1 causes an inflammatory response, which can be beneficial, such as in mounting an immune response to a pathogen, or when overexpressed can lead to other detrimental effects of
20 inflammation.

The cellular effects of IL-1 are initiated by the binding of IL-1 to its receptors (IL-1-Rs) on the surface of target cells. The isolation of polynucleotides encoding IL-1-Rs and variant forms of such receptors has been described in U.S. Patent Nos. 4,968,607, 5,081,228, 5,180,812, in PCT Publication No. WO91/18982,
25 and by Sims et al., PNAS, 86, 8946 (1989) (disclosing the p80, type I IL-1 receptor). Processes for purification of IL-1-Rs have also been disclosed in U.S. Patent No. 5,296,592.

Native IL-1-Rs are characterized by distinct extracellular, transmembrane and intracellular domains. The primary purpose of the extracellular domain is to present
30 a binding site for IL-1 on the outside of the cell. When IL-1 is bound to the binding site, a "signal" is transmitted to the inside of the cell through the transmembrane and intracellular domains, indicating that binding has occurred. Transmission or "transduction" of the signal to the inside of the cell occurs by a change in conformation of the transmembrane and/or intracellular domains of the receptor. This signal is "received" by the binding of proteins and other molecules to the intracellular domain of the receptor, resulting in the effects seen upon IL-1 stimulation.

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While IL-1 binding by IL-1-Rs results in beneficial cellular effects, it is often desirable to prevent or deter IL-1 binding from causing other detrimental cellular effects. Although substantial effort has been expended investigating inhibition of IL-1 binding to the extracellular domain of IL-1-Rs, examination of binding of proteins and other molecules to the intracellular domain of IL-1-Rs has received much less attention.

However, ligands which bind to the IL-1-R intracellular domain have yet to be identified. It would be desirable to identify and isolate such ligands to examine their effects upon IL-1-R signal transduction and their use as therapeutic agents for treatment of IL-1-induced conditions. Furthermore, identification of such ligands would provide a means for screening for inhibitors of IL-1-R/intracellular ligand binding, which will also be useful as anti-inflammatory agents.

SUMMARY OF THE INVENTION

Applicants have for the first time identified novel IL-1-R intracellular ligand proteins and have isolated polynucleotides encoding such ligands. Applicants have also identified certain known proteins which may also bind to the intracellular domain of IL-1-R.

In one embodiment, the present invention provides a composition comprising an isolated polynucleotide encoding a protein having IL-1-R intracellular ligand protein activity. In preferred embodiments, the polynucleotide is selected from the group consisting of:

- (a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 2 to nucleotide 529;
- (b) a polynucleotide comprising a fragment of the nucleotide sequence of SEQ ID NO:1, which encodes a protein having IL-1-R intracellular ligand protein activity;
- (c) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising the amino acid sequence of SEQ ID NO:2;
- (d) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising a fragment of the amino acid sequence of SEQ ID NO:2 and having IL-1-R intracellular ligand protein activity;

(e) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:3 from nucleotide 2 to nucleotide 961;

(f) a polynucleotide comprising a fragment of the nucleotide sequence of SEQ ID NO:3, which encodes a protein having IL-1-R intracellular ligand protein activity;

(g) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising the amino acid sequence of SEQ ID NO:4;

(h) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising a fragment of the amino acid sequence of SEQ ID NO:4 and having IL-1-R intracellular ligand protein activity;

(i) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:5 from nucleotide 2 to nucleotide 754;

(j) a polynucleotide comprising a fragment of the nucleotide sequence of SEQ ID NO:5, which encodes a protein having IL-1-R intracellular ligand protein activity;

(k) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising the amino acid sequence of SEQ ID NO:6;

(l) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising a fragment of the amino acid sequence of SEQ ID NO:6 and having IL-1-R intracellular ligand protein activity; and

(m) a polynucleotide capable of hybridizing under stringent conditions to any one of the polynucleotides specified in (a)-(l), which encodes a protein having IL-1-R intracellular ligand protein activity.

In certain preferred embodiments, the polynucleotide is operably linked to an expression control sequence. The invention also provides a host cell, including bacterial, yeast, insect and mammalian cells, transformed with such polynucleotide compositions.

Processes are also provided for producing an IL-1-R intracellular ligand protein, which comprises:

(a) growing a culture of the host cell transformed with such polynucleotide compositions in a suitable culture medium; and

(b) purifying the IL-1-R intracellular ligand protein from the culture.

The ligand protein produced according to such methods is also provided by the present invention.

5 Compositions comprising a protein having IL-1-R intracellular ligand protein activity are also disclosed. In preferred embodiments the protein comprises an amino acid sequence selected from the group consisting of:

- (a) the amino acid sequence of SEQ ID NO:2;
- (b) fragments of the amino acid sequence of SEQ ID NO:2;
- 10 (c) the amino acid sequence of SEQ ID NO:4;
- (d) fragments of the amino acid sequence of SEQ ID NO:4;
- (e) the amino acid sequence of SEQ ID NO:6; and
- (f) fragments of the amino acid sequence of SEQ ID NO:6;

the protein being substantially free from other mammalian proteins. Such
15 compositions may further comprise a pharmaceutically acceptable carrier.

Compositions comprising an antibody which specifically reacts with such IL-1-R intracellular ligand protein are also provided by the present invention.

Methods are also provided for identifying an inhibitor of IL-1-R intracellular domain binding which comprise:

- 20 (a) combining an IL-1-R intracellular domain protein with an IL-1-R intracellular ligand protein, said combination forming a first binding mixture;
- (b) measuring the amount of binding between the IL-1-R intracellular domain protein and the IL-1-R intracellular ligand protein in the
25 first binding mixture;
- (c) combining a compound with the IL-1-R intracellular domain protein and an IL-1-R intracellular ligand protein to form a second binding mixture;
- (d) measuring the amount of binding in the second binding
30 mixture; and
- (e) comparing the amount of binding in the first binding mixture with the amount of binding in the second binding mixture;

wherein the compound is capable of inhibiting IL-1-R intracellular domain binding when a decrease in the amount of binding of the second binding mixture occurs. In certain preferred embodiments the IL-1-R intracellular ligand protein used in such method comprises an amino acid sequence selected from the group consisting of:

- 5 (a) the amino acid sequence of SEQ ID NO:2;
- (b) fragments of the amino acid sequence of SEQ ID NO:2;
- (c) the amino acid sequence of SEQ ID NO:4;
- (d) fragments of the amino acid sequence of SEQ ID NO:4;
- (e) the amino acid sequence of SEQ ID NO:6;
- 10 (f) fragments of the amino acid sequence of SEQ ID NO:6;
- (g) the amino acid sequence of SEQ ID NO:7; and
- (h) fragments of the amino acid sequence of SEQ ID NO:7.

Compositions comprising inhibitors identified according to such method are also provided. Such compositions may include pharmaceutically acceptable carriers.

- 15 Methods are also provided for preventing or ameliorating an inflammatory condition which comprises administering a therapeutically effective amount of a composition comprising a protein having IL-1-R intracellular ligand protein activity and a pharmaceutically acceptable carrier.

- 20 Other embodiments provide methods of inhibiting IL-1-R intracellular domain binding comprising administering a therapeutically effective amount of a composition comprising a protein having IL-1-R intracellular ligand protein activity and a pharmaceutically acceptable carrier.

- 25 Methods of preventing or ameliorating an inflammatory condition or of inhibiting IL-1-R intracellular domain binding are provided, which comprise administering to a mammalian subject a therapeutically effective amount of inhibitors of IL-1-R intracellular domain binding, are also provided.

Methods of identifying an inhibitor of IL-1-R intracellular domain binding are also provided by the present invention which comprise:

- 30 (a) transforming a cell with a first polynucleotide encoding an IL-1-R intracellular domain protein, a second polynucleotide encoding an IL-1-R intracellular ligand protein, and at least one reporter gene, wherein the expression of the reporter gene is regulated by the binding of the IL-1-R

intracellular ligand protein encoded by the second polynucleotide to the IL-1-R intracellular domain protein encoded by the first polynucleotide;

(b) growing the cell in the presence of and in the absence of a compound; and

5 (c) comparing the degree of expression of the reporter gene in the presence of and in the absence of the compound;

wherein the compound is capable of inhibiting IL-1-R intracellular domain binding when a decrease in the degree of expression of the reporter gene occurs. In preferred embodiments, the cell is a yeast cell and the second polynucleotide is selected from

10 the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 2 to nucleotide 529;

(b) a polynucleotide comprising a fragment of the nucleotide sequence of SEQ ID NO:1, which encodes a protein having IL-1-R
15 intracellular ligand protein activity;

(c) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising the amino acid sequence of SEQ ID NO:2;

(d) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising a fragment of the amino acid sequence of SEQ ID NO:2
20 and having IL-1-R intracellular ligand protein activity;

(e) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:3 from nucleotide 2 to nucleotide 961;

(f) a polynucleotide comprising a fragment of the nucleotide sequence of SEQ ID NO:3, which encodes a protein having IL-1-R
25 intracellular ligand protein activity;

(g) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising the amino acid sequence of SEQ ID NO:4;

(h) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising a fragment of the amino acid sequence of SEQ ID NO:4
30 and having IL-1-R intracellular ligand protein activity;

(i) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:5 from nucleotide 2 to nucleotide 754;

(j) a polynucleotide comprising a fragment of the nucleotide sequence of SEQ ID NO:5, which encodes a protein having IL-1-R intracellular ligand protein activity;

5 (k) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising the amino acid sequence of SEQ ID NO:6;

(l) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising a fragment of the amino acid sequence of SEQ ID NO:6 and having IL-1-R intracellular ligand protein activity;

10 (m) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising the amino acid sequence of SEQ ID NO:7;

(n) a polynucleotide encoding an IL-1-R intracellular ligand protein comprising a fragment of the amino acid sequence of SEQ ID NO:7 and having IL-1-R intracellular ligand protein activity; and

15 (o) a polynucleotide capable of hybridizing under stringent conditions to any one of the polynucleotides specified in (a)-(n), which encodes a protein having IL-1-R intracellular ligand protein activity.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 depicts an autoradiograph demonstrating the expression of IL-1-R intracellular ligand proteins of the present invention in mammalian cells. The expression of flag-14w, -31w and -19w was detected by an anti-flag antibody, M2, as described below.

Fig. 2 demonstrates the effects of the clone 19w product on JNK1 activation. Top panel: HA-tagged JNK1 was coexpressed in COS cells with either pED flag vector or pED flag-19w. After 48 hr, the cells were treated with different concentrations of IL-1 α for 15 min. JNK1 was isolated by immunoprecipitation with 12CA5 antibody and JNK activity was measured using an immune complex kinase assay with the substrate GST-c-jun (1-79). Middle panel: The expression and recovery of HA-JNK1 from immunoprecipitation was examined by Western blot analysis with 12CA5 antibody. Bottom panel: The expression of clone 19w was detected by Western blot analysis of cell lysate using M2 antibody.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have for the first time identified and isolated novel polynucleotides which encode proteins which bind to the IL-1-R intracellular domain. As used herein "IL-1-R" includes all receptors for interleukin-1. The type I, p80 IL-1-R is the preferred receptor for practicing the present invention.

The sequence of a polynucleotide encoding one such protein is set forth in SEQ ID NO:1 from nucleotide 2 to 529. This polynucleotide has been identified as "clone 19w." The amino acid sequence of the IL-1-R intracellular ligand protein encoded by clone 19w is set forth in SEQ ID NO:2. It is believed that clone 19w is a partial cDNA clone of a longer full length coding sequence. However, as demonstrated herein the protein encoded by clone 19w does bind the intracellular domain of IL-1-R (i.e., has "IL-1-R intracellular ligand protein activity" as defined herein). Clone 19w was deposited with the American Type Culture Collection on March 31, 1995 and given the accession number ATCC 69774. The protein encoded by clone 19w is 176 amino acids in length. No identical or closely related sequences were found using database searches. Therefore, clone 19w encodes a novel protein. However, using an extensive FASTA search, a significant homology to amino acids 330 to 390 of thrombospondin (41% identity in 59 amino acids) is found in the C-terminal portion of the 19w protein. Moreover, a significant homology to the Ca²⁺ binding domain, EF hand of calmodulin (25% in 65 amino acids) is observed in the region between amino acids 40 and 110 of the protein encoded by clone 19w.

The sequence of a polynucleotide encoding another such protein is set forth in SEQ ID NO:3 from nucleotide 2 to 961. This polynucleotide has been identified as "clone 31w." The amino acid sequence of the IL-1-R intracellular ligand protein encoded by clone 31w is set forth in SEQ ID NO:4. It is believed that clone 31w is a partial cDNA clone of a longer full length coding sequence. However, as demonstrated herein the protein encoded by clone 31w does bind the intracellular domain of IL-1-R (i.e., has "IL-1-R intracellular ligand protein activity" as defined herein). Clone 31w was deposited with the American Type Culture Collection on March 31, 1995 and given the accession number ATCC 69775. The protein encoded by clone 31w is 320 amino acids in length. No identical or closely related sequences

were found using BLASTN/BLASTX or FASTA searches. Therefore, clone 31w encodes a novel protein.

The sequence of a polynucleotide encoding another such protein is set forth in SEQ ID NO:5 from nucleotides 2 to 754. This polynucleotide has been identified as "clone 14w." The amino acid sequence of the IL-1-R intracellular ligand protein encoded by clone 14w is set forth in SEQ ID NO:6. It is believed that clone 14w is a partial cDNA clone of a longer full length coding sequence. However, as demonstrated herein the protein encoded by clone 14w does bind the intracellular domain of IL-1-R (i.e., has "IL-1-R intracellular ligand protein activity" as defined herein). Clone 14w was deposited with the American Type Culture Collection on March 31, 1995 and given the accession number ATCC 69773.

The protein encoded by clone 14w is identical to the sequence of amino acids 449 to 700 of calcium activated neutral protease (CANP), with the exception of an amino acid change (Val to Phe) at position 553 of CANP. The sequence of CANP is disclosed in Imajoh et al., Biochemistry 1988, 27, 8122-8128, which is incorporated herein by reference (accession no. A31218). The amino acid sequence of CANP is set forth in SEQ ID NO:7. Based upon this sequence homology, CANP and certain fragments thereof will exhibit IL-1-R intracellular ligand binding activity (as defined herein).

For the purposes of the present application, "IL-1-R intracellular ligand protein" includes proteins which exhibit IL-1-R intracellular ligand protein activity. For the purposes of the present application, a protein is defined as having "IL-1-R intracellular ligand protein activity" when it binds to a protein derived from the IL-1-R intracellular domain. Activity can be measured by using any assay which will detect binding to an IL-1-R intracellular domain protein. Examples of such assays include without limitation the interaction trap assays and assays in which IL-1-R intracellular domain protein which is affixed to a surface in a manner conducive to observing binding, including without limitation those described in Examples 1 and 3. As used herein an "IL-1-R intracellular domain protein" includes the entire intracellular domain or fragments thereof.

Fragments of the IL-1-R intracellular ligand protein which are capable of interacting with the IL-1-R intracellular domain or which are capable of inhibiting

IL-1-R intracellular domain binding (i.e., exhibit IL-1-R intracellular ligand protein activity) are also encompassed by the present invention. Fragments of the IL-1-R intracellular ligand protein may be in linear form or they may be cyclized using known methods, for example, as described in H.U. Saragovi, *et al.*, Bio/Technology 5 10, 773-778 (1992) and in R.S. McDowell, *et al.*, J. Amer. Chem. Soc. 114, 9245-9253 (1992), both of which are incorporated herein by reference. Such fragments may be fused to carrier molecules such as immunoglobulins for many purposes, including increasing the valency of IL-1-R intracellular ligand protein binding sites. For example, fragments of the IL-1-R intracellular ligand protein may be fused 10 through "linker" sequences to the Fc portion of an immunoglobulin. For a bivalent form of the IL-1-R intracellular ligand protein, such a fusion could be to the Fc portion of an IgG molecule. Other immunoglobulin isotypes may also be used to generate such fusions. For example, an IL-1-R intracellular ligand protein - IgM fusion would generate a decavalent form of the IL-1-R intracellular ligand protein 15 of the invention.

The isolated polynucleotide of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman *et al.*, Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the IL-1-R intracellular ligand protein recombinantly. Many suitable expression control 20 sequences are known in the art. General methods of expressing recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means that the isolated polynucleotide of the invention and the expression control sequence are situated within a vector or cell in such a way that the IL-1-R intracellular ligand protein is 25 expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

A number of types of cells may act as suitable host cells for expression of the IL-1-R intracellular ligand protein. Host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human 30 epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of

primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK or Jurkat cells.

The IL-1-R intracellular ligand protein may also be produced by operably linking the isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, California, U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated herein by reference.

Alternatively, it may be possible to produce the IL-1-R intracellular ligand protein in lower eukaryotes such as yeast or in prokaryotes such as bacteria. Potentially suitable yeast strains include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* strains, *Candida*, or any yeast strain capable of expressing heterologous proteins. Potentially suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing heterologous proteins. If the IL-1-R intracellular ligand protein is made in yeast or bacteria, it may be necessary to modify the protein produced therein, for example by phosphorylation or glycosylation of the appropriate sites, in order to obtain the functional IL-1-R intracellular ligand protein. Such covalent attachments may be accomplished using known chemical or enzymatic methods.

The IL-1-R intracellular ligand protein of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a nucleotide sequence encoding the IL-1-R intracellular ligand protein.

The IL-1-R intracellular ligand protein of the invention may be prepared by culturing transformed host cells under culture conditions suitable to express the recombinant protein. The resulting expressed protein may then be purified from such culture (i.e., from culture medium or cell extracts) using known purification processes, such as gel filtration and ion exchange chromatography. The purification of the IL-1-R intracellular ligand protein may also include an affinity column

containing the IL-1-R intracellular domain or other IL-1-R intracellular domain protein; one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; one or more steps involving hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or immunoaffinity chromatography.

Alternatively, the IL-1-R intracellular ligand protein of the invention may also be expressed in a form which will facilitate purification. For example, it may be expressed as a fusion protein, such as those of maltose binding protein (MBP) or glutathione-S-transferase (GST). Kits for expression and purification of such fusion proteins are commercially available from New England BioLab (Beverly, MA) and Pharmacia (Piscataway, NJ), respectively. The IL-1-R ligand protein can also be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope ("Flag") is commercially available from Kodak (New Haven, CT).

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the IL-1-R intracellular ligand protein. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous isolated recombinant protein. The IL-1-R intracellular ligand protein thus purified is substantially free of other mammalian proteins and is defined in accordance with the present invention as an "isolated IL-1-R intracellular ligand protein."

IL-1-R intracellular ligand proteins may also be produced by known conventional chemical synthesis. Methods for constructing the proteins of the present invention by synthetic means are known to those skilled in the art. The synthetically-constructed protein sequences, by virtue of sharing primary, secondary or tertiary structural and/or conformational characteristics with IL-1-R intracellular ligand proteins may possess biological properties in common therewith, including IL-1-R intracellular ligand protein activity. Thus, they may be employed as biologically active or immunological substitutes for natural, purified IL-1-R intracellular ligand proteins in screening of therapeutic compounds and in immunological processes for the development of antibodies.

The IL-1-R intracellular ligand proteins provided herein also include proteins characterized by amino acid sequences similar to those of purified IL-1-R intracellular ligand proteins but into which modification are naturally provided or deliberately engineered. For example, modifications in the peptide or DNA sequences can be made by those skilled in the art using known techniques. Modifications of interest in the IL-1-R intracellular ligand protein sequences may include the replacement, insertion or deletion of a selected amino acid residue in the coding sequence. For example, one or more of the cysteine residues may be deleted or replaced with another amino acid to alter the conformation of the molecule. Mutagenic techniques for such replacement, insertion or deletion are well known to those skilled in the art (see, e.g., U.S. Patent No. 4,518,584).

Other fragments and derivatives of the sequences of IL-1-R intracellular ligand proteins which would be expected to retain IL-1-R intracellular ligand protein activity in whole or in part and may thus be useful for screening or other immunological methodologies may also be easily made by those skilled in the art given the disclosures herein. Such modifications are believed to be encompassed by the present invention.

IL-1-R intracellular ligand protein of the invention may also be used to screen for agents which are capable of inhibiting or blocking binding of an IL-1-R intracellular ligand protein to the intracellular domain of IL-1-R, and thus may act as inhibitors of IL-1-R intracellular domain binding and/or IL-1 activity. Binding assays using a desired binding protein, immobilized or not, are well known in the art and may be used for this purpose using the IL-1-R intracellular ligand protein of the invention. Examples 1 and 3 describe examples of such assays. Appropriate screening assays may be cell-based or cell-free. Alternatively, purified protein based screening assays may be used to identify such agents. For example, IL-1-R intracellular ligand protein may be immobilized in purified form on a carrier and binding to purified IL-1-R intracellular domain may be measured in the presence and in the absence of potential inhibiting agents. A suitable binding assay may alternatively employ purified IL-1-R intracellular domain immobilized on a carrier, with a soluble form of a IL-1-R intracellular ligand protein of the invention. Any

IL-1-R intracellular ligand protein may be used in the screening assays described above.

In such a screening assay, a first binding mixture is formed by combining IL-1-R intracellular domain protein and IL-1-R intracellular ligand protein, and the amount of binding in the first binding mixture (B_0) is measured. A second binding mixture is also formed by combining IL-1-R intracellular domain protein, IL-1-R intracellular ligand protein, and the compound or agent to be screened, and the amount of binding in the second binding mixture (B) is measured. The amounts of binding in the first and second binding mixtures are compared, for example, by performing a B/B_0 calculation. A compound or agent is considered to be capable of inhibiting IL-1-R intracellular domain binding if a decrease in binding in the second binding mixture as compared to the first binding mixture is observed. The formulation and optimization of binding mixtures is within the level of skill in the art. Such binding mixtures may also contain buffers and salts necessary to enhance or to optimize binding, and additional control assays may be included in the screening assay of the invention.

Alternatively, appropriate screening assays may be cell based. For example, the binding or interaction between an IL-1-R ligand protein and the IL-1-R intracellular domain can be measured in yeast as described below in Examples 1 and 3.

Compounds found to reduce, preferably by at least about 10%, more preferably greater than about 50% or more, the binding activity of IL-1-R intracellular ligand protein to IL-1-R intracellular domain may thus be identified and then secondarily screened in other binding assays, including *in vivo* assays. By these means compounds having inhibitory activity for IL-1-R intracellular domain binding which may be suitable as anti-inflammatory agents may be identified.

Isolated IL-1-R intracellular ligand protein may be useful in treating, preventing or ameliorating inflammatory conditions and other conditions, such as osteoporosis, colitis, myelogenous leukemia, diabetes, wasting and atherosclerosis. Isolated IL-1-R intracellular ligand protein may be used itself as an inhibitor of IL-1-R intracellular domain binding or to design inhibitors of IL-1-R intracellular domain binding. Inhibitors of binding of IL-1-R intracellular ligand protein to the IL-1-R

intracellular domain ("IL-1-R intracellular binding inhibitors") are also useful for treating such conditions.

The present invention encompasses both pharmaceutical compositions and therapeutic methods of treatment or use which employ isolated IL-1-R intracellular
5 ligand protein and/or binding inhibitors of IL-1-R intracellular binding.

Isolated IL-1-R intracellular ligand protein or binding inhibitors (from whatever source derived, including without limitation from recombinant and non-recombinant cell lines) may be used in a pharmaceutical composition when combined with a pharmaceutically acceptable carrier. Such a composition may also contain (in
10 addition to IL-1-R intracellular ligand protein or binding inhibitor and a carrier) diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of
15 administration. The pharmaceutical composition of the invention may also contain cytokines, lymphokines, or other hematopoietic factors such as M-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, G-CSF, Meg-CSF, stem cell factor, and erythropoietin. The pharmaceutical composition may further contain other anti-inflammatory agents. Such additional factors and/or agents
20 may be included in the pharmaceutical composition to produce a synergistic effect with isolated IL-1-R intracellular ligand protein or binding inhibitor, or to minimize side effects caused by the isolated IL-1-R intracellular ligand protein or binding inhibitor. Conversely, isolated IL-1-R intracellular ligand protein or binding inhibitor may be included in formulations of the particular cytokine, lymphokine, other
25 hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent to minimize side effects of the cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which isolated IL-1-R intracellular ligand protein or binding inhibitor is
30 combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers in aqueous solution. Suitable lipids

for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 5 4,837,028; and U.S. Patent No. 4,737,323, all of which are incorporated herein by reference.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, i.e., treatment, healing, prevention 10 or amelioration of an inflammatory response or condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in 15 combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of isolated IL-1-R intracellular ligand protein or binding inhibitor is administered to a mammal having a condition to be treated. Isolated IL-1-R intracellular ligand protein or binding inhibitor may be administered 20 in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines or other hematopoietic factors. When co-administered with one or more cytokines, lymphokines or other hematopoietic factors, isolated IL-1-R intracellular ligand protein or binding inhibitor may be administered either simultaneously with the 25 cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering isolated IL-1-R intracellular ligand protein or binding inhibitor in combination with cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors. 30 Administration of isolated IL-1-R intracellular ligand protein or binding inhibitor used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral

ingestion, inhalation, or cutaneous, subcutaneous, or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of isolated IL-1-R intracellular ligand protein or binding inhibitor is administered orally, isolated IL-1-R intracellular
5 ligand protein or binding inhibitor will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% isolated IL-1-R intracellular ligand protein or binding inhibitor, and preferably from about 25
10 to 90% isolated IL-1-R intracellular ligand protein or binding inhibitor. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols
15 such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of isolated IL-1-R intracellular ligand protein or binding inhibitor, and preferably from about 1 to 50% isolated IL-1-R intracellular ligand protein or binding inhibitor.

20 When a therapeutically effective amount of isolated IL-1-R intracellular ligand protein or binding inhibitor is administered by intravenous, cutaneous or subcutaneous injection, isolated IL-1-R intracellular ligand protein or binding inhibitor will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having
25 due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to isolated IL-1-R intracellular ligand protein or binding inhibitor, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated
30 Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

5 The amount of isolated IL-1-R intracellular ligand protein or binding inhibitor in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of isolated IL-1-R intracellular ligand protein or binding inhibitor with which to treat each individual patient. Initially, the attending physician will administer low doses of isolated IL-1-R intracellular ligand protein or binding inhibitor and observe the patient's response. Larger doses of isolated IL-1-R intracellular ligand protein or binding inhibitor may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.1 μ g to about 100 mg of isolated IL-1-R intracellular ligand protein or binding inhibitor per kg body weight.

15 The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the isolated IL-1-R intracellular ligand protein or binding inhibitor will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

25 Isolated IL-1-R intracellular ligand protein of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the IL-1-R intracellular ligand protein and which may inhibit IL-1-R intracellular domain binding. Such antibodies may be obtained using either the entire IL-1-R intracellular ligand protein or fragments of IL-1-R intracellular ligand protein as an immunogen. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Methods for synthesizing such peptides are known in the art, for example, as in R.P. Merrifield, J. Amer.Chem.Soc. 85, 2149-2154 (1963); J.L. Krstenansky, *et al.*, FEBS Lett. 211, 10 (1987).

Monoclonal antibodies binding to IL-1-R intracellular ligand protein or to complex carbohydrate moieties characteristic of the IL-1-R intracellular ligand glycoprotein may be useful diagnostic agents for the immunodetection of IL-1-R ligand protein.

5 Neutralizing monoclonal antibodies binding to IL-1-R intracellular ligand protein or to complex carbohydrates characteristic of IL-1-R intracellular ligand glycoprotein may also be useful therapeutics for both inflammatory conditions and also in the treatment of some forms of cancer where abnormal expression of IL-1-R intracellular ligand protein is involved. These neutralizing monoclonal antibodies are
10 capable of blocking the signaling function of the IL-1-R intracellular ligand protein. By blocking the binding of IL-1-R intracellular ligand protein, certain biological responses to IL-1 are either abolished or markedly reduced. In the case of cancerous cells or leukemic cells, neutralizing monoclonal antibodies against IL-1-R intracellular ligand protein may be useful in detecting and preventing the metastatic
15 spread of the cancerous cells, which may be mediated by the IL-1-R intracellular ligand protein.

Due to the similarity of its sequence to SEQ ID NO:6, CANP and fragments thereof which bind to the IL-1-R intracellular domain are proteins having IL-1-R intracellular ligand protein activity as defined herein. As a result, they are also
20 useful in pharmaceutical compositions, for treating inflammatory conditions and for inhibiting IL-1-R intracellular domain binding as described above for IL-1-R intracellular ligand proteins generally.

EXAMPLE 1 CLONING OF IL-1-R INTRACELLULAR LIGAND PROTEIN ENCODING POLYNUCLEOTIDE

25 A yeast genetic selection method, the "interaction trap" [Gyuris et al, Cell 75:791-803, 1993, which is incorporated herein by reference], was used to screen
30 WI38 and HeLa cell cDNA libraries (preparation, see below) for proteins that interact with IL-1-R-1c, the cytoplasmic portion (intracellular domain) of the interleukin-1 receptor p80, or type I. The IL-1-R-1c DNA, encoding amino acids 340 to 552 of the type I IL-1 receptor, was obtained via the polymerase chain

reaction (PCR) of a human WI38 cell cDNA library. This IL-1-R-1c DNA was then cloned into pEG202 by an EcoRI site, generating the bait plasmid, pEG202-IL-1-R-1c. This plasmid contains the HIS3 selectable marker, and expression of the bait, the LexA-IL-1-R-1c fusion protein, is from the strong
5 constitutive ADH1 promoter. To create the reporter strain carrying the bait protein, yeast strain EGY48, containing the reporter sequence LexAop-Leu2 in place of the chromosomal LEU2, was transformed with pEG202-IL-1-R-1c and pSH18-34 (Ura+), which carries another reporter sequence, LexAop-lacZ. For screening cDNAs encoding proteins that interact with IL-1-R-1c, the expression vector pJG4-5 (TRP1),
10 containing either a WI38 or HeLa cell cDNA library (see below for the cDNA library construction), was transformed into the above strain (EGY48/pEG202-IL-1-R-1c/pSH18-34) according to the method described by Gietz et al., Nucleic Acids Res., 20, 1425, 1992.

The bait used in obtaining clones 14w, 19w and 31w was constructed by
15 cloning the DNA sequences encoding amino acids 477 to 527 of IL-1 receptor p80 into the EcoRI and NotI sites of EG202. The resulting plasmid was named EG202-IL1R (477-527). This region of the IL-1 receptor is believed to be essential for signaling.

20 **cDNA Library Construction:**

WI38 cell cDNA library: Double stranded cDNA was prepared from 3 µg of WI38 mRNA using reagents provided by the Superscript Choice System (Gibco/BRL, Gaithersburg, MD) with the following substitutions: the first strand synthesis was primed using an oligo dT/XhoI primer/linker, and the dNTP mix was
25 substituted with a mix containing methyl dCTP (Stratagene, LaJolla, CA). The cDNA was modified at both ends by addition of an EcoRI/NotI/SalI adapter linker and subsequently digested with XhoI. This produced cDNA molecules possessing an EcoRI/NotI/SalI overhang at the 5' end of the gene and an XhoI overhang at the 3' end. These fragments were then ligated into the yeast expression/fusion vector
30 pJG4-5 (Gyuris et al., Cell, 75, 791-803, 1993), which contains at its amino terminus, the influenza virus HA1 epitope tag, the B42 acidic transcription activation domain, and the SV40 nuclear localization signal, all under the control of the

galactose-dependent GAL1 promoter. The resulting plasmids were then electroporated into DH10B cells (Gibco/BRL). A total of 7.1×10^6 colonies were plated on LB plates containing 100 ug/ml of ampicillin. These *E.coli* were scraped, pooled, and a large scale plasmid prep was performed using the Wizard Maxi Prep kit (Promega, Madison, WI), yielding 3.2mg of supercoiled plasmid DNA.

HeLa cell cDNA: HeLa cell cDNA preparation methods are described in Gyuris et al., Cell, 75, 791-803, 1993, which is incorporated herein by reference.

HeLa Cell cDNA Screening Results:

2 x 10^5 transformants were obtained on glucose Ura⁻His⁻Trp⁻ plates. These transformants were pooled and resuspended in a solution of 65% glycerol, 10mM Tris-HCl (pH 7.5), 10 mM MgCl₂ and stored at -80°C in 1mL aliquots. For screening purposes, aliquots of these were diluted 10-fold into Ura⁻His⁻Trp⁻ CM dropout gal/raff medium (containing 2% galactose, 1% raffinose), which induces the expression of the library encoded proteins, and incubated at 30°C for 4 hours. 2 x 10^6 colony forming units (CFUs) were then plated on standard 10cm galactose X-Gal Ura⁻His⁻Trp⁻Leu⁻ plates at a density of 2 x 10^5 CFU/plate. After 4 days at 30°C, colonies that were strong LacZ⁺ were chosen for further processing. In order to test if the Leu⁺/LacZ⁺ phenotype was due to the library-encoded protein, the galactose dependency of the phenotype was tested. Expression of the library-encoded proteins was turned off by growth on glucose Ura⁻His⁻Trp⁻ master plates and then retested for galactose-dependency on glucose Ura⁻His⁻Trp⁻Leu⁻, galactose Ura⁻His⁻Trp⁻Leu⁻, glucose X-Gal Ura⁻His⁻Trp⁻, and galactose X-Gal Ura⁻His⁻Trp⁻ plates. Of these, many colonies showed galactose-dependent growth on Leu⁻ plates and galactose-dependent blue color on X-Gal-containing medium (LacZ⁺ phenotype). Total yeast DNA was prepared from these colonies according to the method described previously (Hoffman and Winston, 1987). In order to analyze the cDNA sequences, PCR reactions were performed using the above yeast DNA as a template and oligo primers specific for the vector pJG4-5, flanking the cDNA insertion point. PCR products were purified (Qiagen PCR purification kit), subjected to restriction digest with the enzyme HaeIII. run on 1.8% agarose gels, and the restriction patterns compared. Similar and identical restriction patterns were grouped and representatives of each group were

sequenced and compared to Genbank and other databases to identify any sequence homologies.

WI38 Cell cDNA Screening Results:

5 This screen was performed as above with the following exceptions: 1) 1×10^6 transformants were obtained on glucose Ura⁻His⁻Trp⁻ plates and pooled. 2) 11×10^6 CFU were screened. Of these, 0.5% were Leu⁺ and of those, 1% were LacZ⁺. This gave a frequency of 50 double positives per 10^6 transformants screened. Colonies, exhibiting a strong LacZ⁺ phenotype (as judged by the strength of blue color on
10 X-Gal containing medium), were chosen for further processing. Clones with the strongest LacZ⁺ phenotype were chosen for further specificity tests as described above.

A WI38 cDNA library was transformed into the reporter strain (EGY48/pSH18-34) containing the bait plasmid EG202-IL1R (477-527). 1.3 million
15 primary transformants were harvested and 7 million colonies were screened. 192 galactose-dependent colonies were isolated. Among these, 51 clones were bait specific (*i.e.*, interacted specifically with the original bait, but not with an unrelated bait, bicoid). These clones were then subjected to DNA sequence analysis. Clones
19w was isolated 6, times, clone 31w twice, and clone 14w once.

20 ..

EXAMPLE 2

EXPRESSION OF THE IL-1-R INTRACELLULAR LIGAND PROTEIN

cDNAs encoding IL-1-R intracellular ligand proteins were released from the
25 pJG4-5 vector with the appropriate restriction enzymes. For example, EcoRI and XhoI were used to release cDNA from the relevant clone. Where the restriction sites were also present in the internal sequence of the cDNA, PCR was performed to obtain the cDNA. These cDNAs were then cloned into various expression vectors. These included pGEX (Pharmacia) or pMAL (New England Biolabs) for expression
30 as a GST (Glutathione-S-transferase) or MBP (maltose binding protein) fusion protein in *E. coli*, a pED-based vector for mammalian expression, and pVL or pBlueBacHis (Invitrogen) for baculovirus/insect expression. For the immunodetection of IL-1-R intracellular ligand expression in mammalian cells, an

epitope sequence, "Flag," was inserted into the translational start site of the pED vector, generating the pED-Flag vector. cDNAs were then inserted into the pED-Flag vector. Thus, the expression of cDNA from pED-Flag yields a protein with an amino terminal Met, followed by the "Flag" sequence, Asp-Tyr-Lys-Asp-Asp-Asp-Lys.

5 Standard DEAE-Dextran or lipofectamine methods were used to transfect COS or CHO dukx cells. Immunodetection of Flag-tagged proteins was achieved using the M2 antibody (Kodak). Moreover, an immunoaffinity column using the M2 antibody, followed by elution with the "Flag" peptide, can be used for the rapid purification of the flag-tagged protein. Similarly, affinity purification of GST- , MBP- or

10 His-tagged fusion proteins can be performed using glutathione, amylose, or nickel columns. Detailed purification protocols are provided by the manufacturers. For many fusion proteins, the IL-1-R intracellular ligand can be released by the action of thrombin, factor Xa, or enterokinase cleavage. In the case where highly purified material is required, standard purification procedures. such as ion-exchange,

15 hydrophobic, and gel filtration chromatography will be applied in addition to the affinity purification step.

Fig. 1 depicts an autoradiograph demonstrating the expression of IL-1-R intracellular ligand proteins in mammalian cells. Fig. 1 shows the results of expression of Flag-14w, -19w and -31w in COS cells. COS cells were transfected

20 with either pED-Flag (vector control), Flag-14w, -19w or -31w plasmid by the lipofectamine method. Thirty µg of each cell lysate were prepared and subjected to 4-20% SDS gel electrophoresis, followed by Western blot analysis using M2 antibody (Kodak). A Flag-containing protein, Flag-BAP (Kodak). was also loaded as a standard. The bands in the Flag-14w, -19w and -31w indicate significant expression

25 of the respective IL-1-R intracellular ligand proteins.

EXAMPLE 3

ASSAYS OF IL-1-R INTRACELLULAR DOMAIN BINDING

30 Two different methods were used to assay for IL-1-R intracellular ligand protein activity. The first assay measures binding in the yeast strain in "interaction trap," the system used here to screen for IL-1-R-1c interacting proteins. In this system, the expression of reporter genes from both LexAop-Leu2 and LexAop-LacZ

relies on the interaction between the bait protein, in this case IL-1-R-1c, and the prey, the IL-1-R intracellular ligand. Thus, one can measure the strength of the interaction by the level of Leu2 or LacZ expression. The most simple method is to measure the activity of the LacZ encoded protein, β -galactosidase. This activity can be judged by the degree of blueness on the X-Gal containing medium or filter. For the quantitative measurement of β -galactosidase activity, standard assays can be found in "Methods in Yeast Genetics" Cold Spring Harbor, New York, 1990 (by Rose, M.D., Winston, F., and Hieter, P.).

The second assay for measuring binding is a cell-free system. An example of a typical assay is described below. Purified MBP-IL-1-R-1c fusion protein (2 μ g) was mixed with glutathione-Sepharose 4 B beads bound with a GST-IL-1-R-1c intracellular ligand for 2 hour at 4°C. The mixture was then centrifuged to separate bound (remained with the beads) and unbound (remained in the supernatant) MBP-IL-1-R-1c. After extensive washing, the bound MBP-IL-1-R-1c was eluted with glutathione and detected by Western blot analysis using an MBP antibody. The IL-1-R-1c or the intracellular ligand can also be immobilized on other solid supports, such as on plates or fluorobeads. The binding can then be measured using ELISA or SPA (scintillation proximity assay).

EXAMPLE 4 CHARACTERIZATION OF IL-1-R INTRACELLULAR LIGAND PROTEIN

Mapping the interaction site in IL-1-R-1c

Many of the key amino acids for IL-1-R signaling have been determined by site-directed mutagenesis (Heguy et al., 1992, JBC, 267, 2605-2609). These amino acids are conserved between IL-1-R and the Drosophila Toll protein, which is required for transducing dorsoventral positional information to cells in the developing embryo. In order to test if the IL-1-R intracellular proteins interact with these residues, these residues were mutagenized and the ability of the mutant protein to interact with the intracellular ligand in the "interaction trap" system was tested. Mutations that abolish IL-1R signaling were introduced into the original bait plasmid,

EG202-IL-1R (477-527) (with following amino acid substitutions: F513A, W514A, K515R, R518K, and Y519S) and the ability of the IL-1R intracellular ligands to interact with these mutant proteins was tested in the interaction trap. EGY48 carrying pSH18-34 (lexAop-LacZ) were cotransformed with two plasmids: one carrying 14w, 19w or 31w; the other with bait, EG202-IL1R (477-527), either wild-type or one of the mutants. transformants were then streaked onto CM ura^{his}trp⁻ plates containing galactose/raffinose and β -gal. The strength of interaction (as indicated by the number of "+" signs) was judged by the blueness in the plates (indicator of LacZ expression). The results are summarized in Table I.

10

Table I

bait →	WT	F513A	W514A	K515R	R518K	Y519S
clone ↓						
14w	+++	++	+	+++	---	-
19w	+	-	+	++	-	-
31w	++	-	-	+++	-	-

15

Clone 14w interacted with mutant baits W514A and Y519S much more weakly than with wild-type bait. Clone 19w interacted differentially with wild-type and many of the mutant baits. It appeared to interact with the mutant bait K515R more strongly than with wild-type, while reduced interaction was observed with mutant baits F513R, R518K and Y519S. The interaction of clone 31w was significantly reduced by mutations F513A, W514A, R518K and Y519S. The change in the interaction strength by these mutations suggests that these residues are the site(s) of interaction. therefore, these data suggest that clones 14w, 19w and 31w interact with many of the signaling residues and may play a role in IL-1R signaling

25

Effect on the IL-1-mediated response

The effect of the IL-1-R intracellular ligands on the IL-1-mediated response can be evaluated in cells overexpressing the ligands. A number of IL-1 mediated

30

responses, including transient or prolonged responses, can be measured. For example, IL-1-induced kinase activity toward either MBP (myelin basic protein) or the N-terminus (amino acids 1-79) of c-jun can be measured in COS cells or CHO cells either transiently or stably overexpressing IL-1R intracellular ligand proteins.

5 Alternatively, other functional assays, such as the induction of gene expression or PGE₂ production after prolonged incubation with IL-1, can also be used to measure the IL-1 mediated response. Conversely, the significance of the IL-1-R intracellular ligand proteins in IL-1 signaling can be established by lowering or eliminating the expression of the ligands. These experiments can be performed using antisense
10 expression or transgenic mice.

IL-1 mediated JNK (c-jun NH₂-terminal kinase, Derjard et al., Cell 1994, 76, 1025-1037) activation was used to study the effect of the IL-1R intracellular ligands on IL-1 signaling. COS cells were transfected with both pEDflag plasmid containing one of the clones (*e.g.*, 19w) and HA-JNK1 plasmid by the DEAE-dextran method.
15 48 hrs after transfection, cells were starved in 0.1% BSA for 1 hr and treated with various amounts of IL-1 α for 15 min. Cells were then lysed, centrifuged and immunoprecipitated with anti-HA monoclonal antibody, 12CA5 (Boehringer Mannheim). JNK activity was performed at 30°C for 20 min using 5 μ g GST-c-jun (1-79 amino acids), 20 μ M ATP, and 5 μ Ci [γ -³²P]ATP in 40 μ l of kinase buffer (25
20 mM HEPES, pH 7.5, 20 mM MgCl₂, 20 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 2 mM DTT). The reactions were terminated using laemmli sample buffer and the products were resolved by SDS-PAGE (4-20%).

As shown in Fig. 2, expression of clone 19w stimulated JNK activity in all IL-1 concentrations tested as compared to the pED flag vector transfected cells. It
25 also enhanced JNK activity even in the absence of IL-1. These data strongly suggest that clone 19w, through its interaction with the signaling domain of IL-1 receptor (*i.e.*, amino acids 477-527 of IL-1R), may indeed participate in the signaling event.

Enzymatic or functional assays

30 The signal transduction events initiated by IL-1 binding to its receptor are still largely unknown. However, one major result of IL-1 binding is the stimulation of cellular serine/threonine kinase activity. In addition, IL-1 has been shown to stimulate

